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RNA Polymerase II Interacts with the Promoter Region of the Noninduced *hsp70* Gene in *Drosophila melanogaster* Cells

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By using a protein-DNA cross-linking method (D. S. Gilmour and J. T. Lis, Mol. Cell. Biol. 5:2009–2018, 1985), we examined the in vivo distribution of RNA polymerase II on the *hsp70* heat shock gene in *Drosophila melanogaster* Schneider line 2 cells. In heat shock-induced cells, a high level of RNA polymerase II was detected on the entire gene, while in noninduced cells, the RNA polymerase II was confined to the 5′ end of the *hsp70* gene, predominantly between nucleotides −12 and +65 relative to the start of transcription. This association of RNA polymerase II was apparent whether the cross-linking was performed by a 10-min UV irradiation of chilled cells with mercury vapor lamps or by a 40-μs irradiation of cells with a high-energy xenon flash lamp. We hypothesize that RNA polymerase II has access to, and a high affinity for, the promoter region of this gene before induction, and this poised RNA polymerase II may be critical in the mechanism of transcription activation.

The *Drosophila melanogaster hsp70* promoter region is structurally complex even in noninduced cells. Nuclease-hypersensitive regions include an interval upstream of the transcription start, as well as an interval immediately downstream of the transcription start, which has a peak of sensitivity at position +42 (8, 32, 33). These features indicate the absence of standard nucleosomes and suggest the presence of nonhistone proteins (10, 11). Wu (33) discovered that a barrier to exonuclease III digestion exists in the −40 to −12 interval of the *hsp70* promoter in the nuclei of noninduced cells. This barrier may be the result of a TATA box-binding protein, which has been partially purified and characterized by in vitro binding assays (25). Inspection of the pattern of exonuclease digestion (33) reveals another barrier 65 base pairs downstream of the transcription initiation site. Thus, the start of the *hsp70* transcription unit may also be associated with protein. In the present paper, we discuss evidence obtained with a protein-DNA cross-linking method (13–15) which leads us to conclude that RNA polymerase II is part of this promoter complex in noninduced cells.

MATERIALS AND METHODS

The protein-DNA cross-linking method was executed as previously described by Gilmour and Lis (14) for the experiments presented in Fig. 1 and 2. Antiserum against the 215,000-dalton subunit of RNA polymerase II (30) was used to recover RNA polymerase II-DNA adducts, and antiserum against *Escherichia coli* RNA polymerase (13) was used to assess nonspecific background. Experiments in Fig. 3 and 4 had the following modifications. Ten-milliliter portions of Schneider line 2 cells (26) were transferred from a spinner flask to a rectangular, siliconized glass dish (6 by 8 cm) and irradiated for either four flashes separated by 1-s intervals or a single flash from a Xenon lamp (model N734; Xenon Corp.). For the experiment with four flashes, the lamp was positioned 10 cm above the cells, whereas for the single-flash experiment the lamp was positioned 0.5 in. (ca. 1.3 cm) above the cells. Power for the lamp was provided by a Xenon model 457 micropulser set at 7,000 V. A trigger wire was attached to the cathode end of the lamp and then extended along the length of the lamp. A 60-μH inductor was in series with the lamp, providing sufficient inductance so that the lamp did not explode at these power levels. By measurement of the current flow with an oscilloscope, this configuration was found to produce a 40-μs pulse of light.

After irradiation, DNA and protein-DNA adducts were isolated from the cells on CsCl gradients. The adducts were dialyzed into 0.2% Sarkosyl (CIBA-GEIGY Corp.)–10 mM Tris chloride (pH 8.0)–1 mM EDTA and then diluted into buffers suitable for restriction enzyme digestion but with the addition of 0.1% Sarkosyl and 0.5% Nonident P-40. After restriction digestion and incubation with antibodies, immune complexes were collected with Formalin-fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring). A 10-μl volume of a 20% (vol/vol) suspension was used per 1 μl of antiserum. Before their use, the Formalin-fixed *S. aureus* cells were washed four times with 1% sodium dodecyl sulfate–50 mM $NaHCO_3$ and twice with 0.5% Sarkosyl–10 mM Tris chloride, pH 7.4–1 mM EDTA. These washes, which do not reduce the binding capacity of the *S. aureus* cells (data not shown), extract DNA from the Formalin-fixed *S. aureus* cells that would otherwise copurify with the immunoprecipitated DNA. The *S. aureus* cell-immune complexes were washed (15), and DNAs were isolated from the immunoprecipitates and from a fraction of the "total" as previously described (14) and analyzed by Southern blotting (15, 27).

RESULTS

A higher level of RNA polymerase II is associated with the 5′ half than the 3′ half of the *hsp70* gene in noninduced cells. The photo-cross-linking method provides a measure of the in vivo concentration of RNA polymerase II on specific DNA restriction fragments (13–15). The cross-links between protein and DNA are generated by irradiating cells with UV light, and protein-DNA adducts are purified by density gradient centrifugation. The DNA is cleaved with restriction enzymes, and adducts containing RNA polymerase II are immunoprecipitated with antiserum to RNA polymerase II. The DNA that coprecipitates is purified and characterized by

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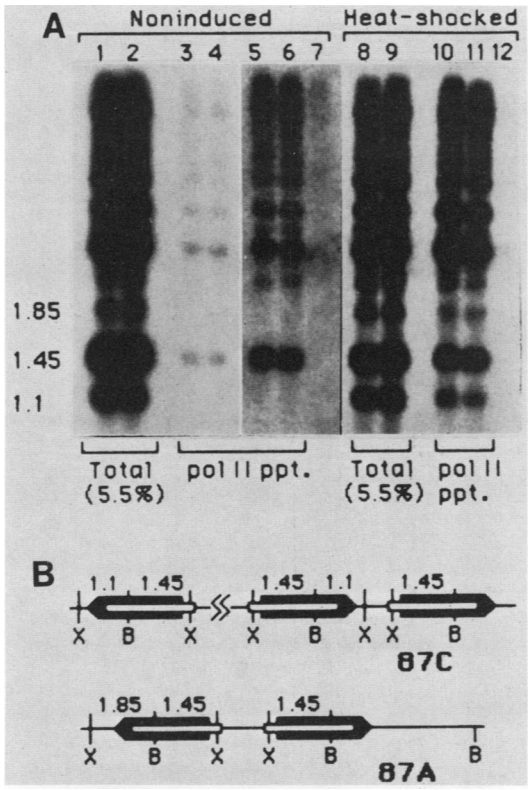

FIG. 1. RNA polymerase II is preferentially associated with the 5′ half of the *hsp70* gene in noninduced cells. (A) Lanes 1 and 2 are 5.5% of the totals from noninduced cells, and lanes 3 and 4 are the immunoprecipitates from these cells. Lanes 5 and 6 are a longer exposure of lanes 3 and 4. Lane 7 is the anti-*E. coli* RNA polymerase immunoprecipitate from noninduced cells. Lanes 8 and 9 are 5.5% of the totals from heat shock-induced cells, and lanes 10 and 11 are the immunoprecipitates from these cells. Lane 12 is the anti-*E. coli* RNA polymerase immunoprecipitate from heat shock-induced cells. Samples in lanes 2, 4, 6, 9, and 11 are derived from samples that were treated with the RNases before the addition of antiserum. The numbers on the left of the autoradiograms indicate fragment size (in kilobases). DNA and protein-DNA complexes from 1.6×10^8 UV-irradiated, noninduced cells and 1.6×10^8 UV-irradiated, heat shock-induced cells were prepared and restriction cut with *Bam*HI and *Xho*I (14). One-third of each sample (5.3×10^7) was then treated with 50 μg each of RNases A, T1, and H per ml for 45 min at 37°C. The RNase-treated samples and half of the DNA not treated with RNases (5.3×10^7) were immunoprecipitated with antiserum against the 215,000-dalton subunit of RNA polymerase II (pol II ppt.). The remaining half of the DNA, which had not been treated with RNases (5.3×10^7), was immunoprecipitated with antiserum to *E. coli* RNA polymerase (lanes 7 and 12). DNA was purified from each immunoprecipitate and fractionated on a 1% agarose gel along with 5.5% of the DNAs purified from the RNA polymerase II immunoprecipitation supernatants (total) (14). All the DNAs were blotted to a nitrocellulose filter. *hsp70* gene sequences were detected with a nick-translated, 2.2-kb *Xba*I restriction fragment purified from plasmid 132E3 (23). (B) Restriction maps of the *hsp70* genes (derived from references 3, 16, 18, and 23). Each *hsp70* transcription unit is depicted as a black arrow with the arrow head at the 3′ end of the gene. Three copies of the gene are present at the 87C locus, and two copies of the gene are present at the 87A locus. The break between the first *hsp70* gene at 87C and the second two represents approximately 30 kb of DNA. Restriction enzymes are as follows: B, *Bam*HI; X, *Xho*I. The numbers above the arrows are the sizes (in kilobases) of the fragments bracketed by the restriction sites. The region of homology between the nick-translated probe and the *hsp70* gene is depicted by the white rectangles.

DNA blotting (27). The enrichment or depletion of a particular sequence in the immunoprecipitate is quantified by comparing the amount of an immunoprecipitated fragment with the amount of the same fragment remaining in a portion of the supernatant.

Figure 1A (lanes 10 and 11) shows that RNA polymerase II cross-links to DNA fragments derived from both the 5′ and 3′ halves of the *hsp70* gene in heat shock-induced cells. Three fragments are products of complete digestion (Fig. 1B): the 1.1-kilobase (kb) fragment corresponds to the 3′ half of two of the *hsp70* genes located at the cytogenetic locus 87C, the 1.85-kb fragment corresponds to the 3′ half of one of the *hsp70* genes located at 87A, and the 1.45-kb fragment corresponds to the 5′ half of all five of the *hsp70* genes (23; D. S. Gilmour, Ph.D. thesis, Cornell University, Ithaca, N.Y., 1984). Although the digests are sometimes incomplete due to the presence of the detergents Sarkosyl and Nonident P-40 which are included in our purification of protein-DNA adducts, we can determine the percentage of any restriction

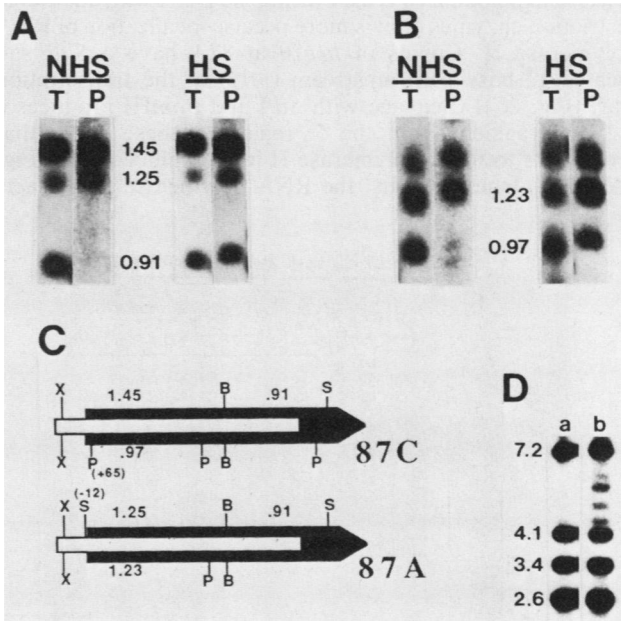

FIG. 2. Detection of RNA polymerase II at the region of the *hsp70* promoter in noninduced cells. (A) Samples of DNA and protein-DNA complexes, each from 1.8×10^8 UV-irradiated, noninduced cells (NHS) or 1.8×10^8 UV-irradiated, heat shock-induced cells (HS), were restriction cut with *Bam*HI, *Xho*I, and *Sal*I. RNA polymerase II-DNA adducts were purified and analyzed along with 5.5% of the total DNA as in Fig. 1. The autoradiographic exposures of the totals (T) were for 3 h, and the exposures of the immunoprecipitated DNAs (P) from heat shock-induced and from noninduced cells were for 1 and 14 days, respectively. (B) As in panel A, except DNA was digested with *Xho*I, *Bam*I, and *Pvu*II. Sizes of the relevant DNA fragments in panels A and B are indicated in the center. (C) Restriction maps represent the *hsp70* genes residing at loci 87A and 87C. Genes are depicted by the black arrows (transcription is from left to right), and the region homologous to the nick-translated probe is depicted by the white rectangle. *Xho*I (X), *Bam*HI (B), and *Sal*I (S) maps are shown above, and the *Xho*I, *Bam*HI, and *Pvu*II (P) maps are shown below, each gene. (D) DNA from 10^7, UV-irradiated, heat shock-induced cells was restriction cut with *Xho*I. RNA polymerase II-DNA adducts were purified and *hsp70* sequences were detected as in panel A except that 2% of the total DNA (lane b) accompanies the immunoprecipitated sample (lane a). One *hsp70* gene is represented in each of the bands at 7.2, 4.1, and 3.4 kb, and two *hsp70* genes comprise the band at 2.6 kb.

fragment that is immunoprecipitated by comparison to tracks containing a known percentage of the unprecipitated supernatant (totals).

Much less RNA polymerase II cross-links to the *hsp70* gene in noninduced cells (Fig. 1A, lanes 3 and 4). This is expected, since transcription of the *hsp70* gene is at least 100-fold lower before than after heat shock induction (20, 29). A longer autoradiographic exposure indicates that the RNA polymerase II is clearly detectable on the 1.45-kb fragment from the 5′ halves but not on the 1.1- and 1.85-kb fragments from the 3′ halves of the *hsp70* genes (Fig. 1A, lanes 5 and 6).

The presence of this 5′ fragment in the immunoprecipitate is not an artifact of the precipitation method since it is not precipitated by antiserum to *E. coli* RNA polymerase (Fig. 1A, lane 7). Moreover, the polymerase-DNA linkage is not via an RNA bridge, since prior treatment of the sample with RNases A, T1, and H does not inhibit the immunoprecipitation of the 1.45-kb fragment (Fig. 1A, lane 6).

RNA polymerase II is concentrated on the promoter region of *hsp70* in noninduced cells. Cutting the DNA with additional restriction enzymes allows more precise localization of RNA polymerase II. Copies of *hsp70* at 87A have a *Sal*I site located 12 base pairs upstream (−12) of the transcription start (Fig. 2C). Cleavage with *Sal*I and *Bam*HI produces a 1.25-kb fragment from the 5′ region of these genes that cross-links to RNA polymerase II in noninduced cells (Fig. 2A, NHS, lane P). Thus, the RNA polymerase II interacts

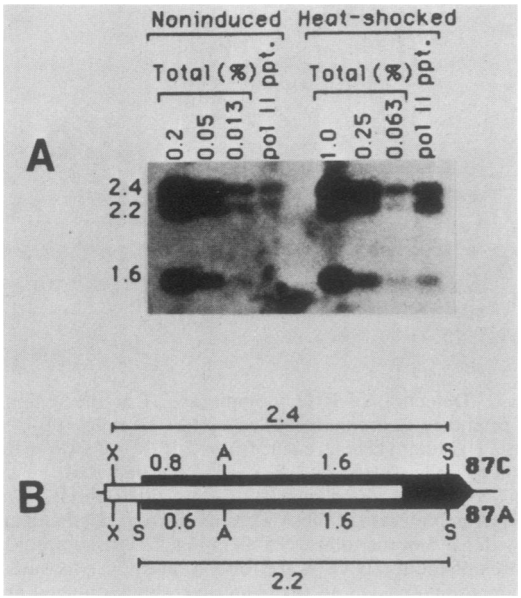

FIG. 3. (A) Detection of RNA polymerase II cross-linked to *hsp70* by irradiation with a xenon flash lamp. Cells (2×10^8) that were noninduced or heat shock induced for 10 min were UV irradiated with four successive flashes spaced at 1-sec intervals as described in the text, and DNA and protein-DNA complexes were prepared. Samples from 10^7 heat shock-induced cells and from 5×10^7 noninduced cells were digested with *Ava*I, *Xho*I, *Sal*I, and *Eco*RI and then immunoprecipitated with antiserum to RNA polymerase II. DNA purified from each immunoprecipitate was fractionated on a 1.5% agarose gel along with indicated amounts of DNA purified from the immunoprecipitation supernatant. *hsp70* sequences were detected by hybridization of a DNA blot of the gel with a nick-translated, 2.2-kb, *Xba*I restriction fragment from plasmid 132E3. Abbreviations are as in Fig. 1. (B) Restriction map symbols are as in Fig. 2C, and *Ava*I (A) was also used.

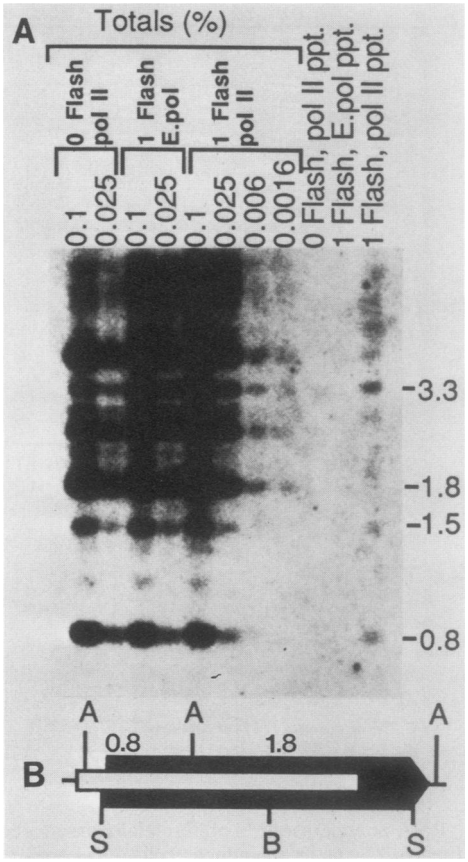

FIG. 4. Detection of RNA polymerase II on the 5′ half of the noninduced *hsp70* gene with a 40-μs irradiation. (A) Noninduced cells (1.5×10^9) were irradiated with a single flash from a xenon lamp (1 Flash). DNA from these cells and DNA from 5×10^8 nonirrradiated cells (0 Flash) were restriction cut with *Ava*I. Half of the DNA sample from irradiated cells (7.5×10^8) was immunoprecipitated with 7.5 μl of antiserum to the 215,000-dalton subunit of RNA polymerase II (pol II ppt.), while the remaining half was immunoprecipitated with 7.5 μl of antiserum to *E. coli* RNA polymerase (E. pol ppt.). All of the DNA sample from nonirradiated cells was immunoprecipitated with 5 μl of antiserum to RNA polymerase II. DNA was purified from each immunoprecipitate, then fractionated on a 1.5% agarose gel along with the indicated amounts of DNA purified from 1% of the immunoprecipitation reaction (totals), and finally blotted to nitrocellulose. *hsp70* sequences were detected with a nick-translated, 2.2-kb *Xba*I restriction fragment purified from plasmid 132E3. (B) Restriction map symbols are as in Fig. 2C, and *Ava*I (A) was also used.

with DNA downstream of −12. All five *hsp70* genes have a 0.91-kb *Bam*HI-*Sal*I fragment from the 3′ region that does not cross-link to RNA polymerase in noninduced cells (Fig. 2A, NHS, lane P). This is in striking contrast to the situation in heat shock-induced cells where this fragment is cross-linked to RNA polymerase II as efficiently as are the 1.25- and 1.45-kb fragments from the 5′ halves of the *hsp70* genes (Fig. 2A, HS, lane P).

Copies of *hsp70* at 87C have a *Pvu*II site located 65 base pairs downstream (+65) of the transcription start (Fig. 2C). Cleavage of these genes with *Pvu*II produces a 0.97-kb fragment from the 5′ half of these genes. In noninduced cells, a very low, but detectable, level of RNA polymerase II cross-links to the 0.97-kb fragment (Fig. 2B, NHS, lane P). (We presume that the lower band [ca. 0.93 kb] of this weakly

hybridizing doublet is a minor product of partial *Xho*I digestion beginning at the +65 *Pvu*II site and extending upstream to a second *Xho*I site [18].) In contrast, a high level of RNA polymerase II cross-links to the 0.97-kb fragment in heat shock-induced cells (Fig. 2B, HS, lane P). The original autoradiogram showed that RNA polymerase II is also cross-linked to the 260-base-pair *Xho*I/*Pvu*II fragment derived from the promoter region of 87C copies of the gene (data not shown), indicating that RNA polymerase II is present upstream of +65 and not simply blocking the restriction site at +65. Thus, RNA polymerase II is predominantly upstream of +65. Although a very low level of RNA polymerase II is cross-linked downstream of +65, no RNA polymerase II is detected downstream of the *Bam*HI site, which is located in the middle of the gene.

The 87C copies of *hsp70* lack the *Sal*I site at −12 but are cut 180 base pairs farther upstream with *Xho*I. Cleavage with *Bam*HI and *Xho*I produces a 1.45-kb fragment from the 5′ end of 87C genes. Because RNA polymerase II cross-links to both this 1.45-kb fragment and the 1.25-kb, *Sal*I-*Bam*HI fragment with nearly equal efficiencies ([Fig. 2A, NHS, lane P], the signal was quantified by analysis of exposures shorter than that shown), we conclude that the majority of the RNA polymerase is downstream of position −12.

In our analyses, we assume that all five genes are regulated in a similar manner. The results (Fig. 2D, lane a) support this assumption. Equal amounts of RNA polymerase II cross-link to three fragments of 7.2, 4.1, and 3.4 kb in length. These each contain a single copy of *hsp70*. The fourth band in this lane is composed of two comigrating 2.6-kb fragments which each contain a single copy of *hsp70* and which together produce a signal approximately twice that of the other three bands.

Cross-linking of RNA polymerase II to the 5′ end of *hsp70* with very short pulses of UV light. From the experiments presented above, we cannot rule out the possibility that the RNA polymerase found at the promoter may be a consequence of induction during the UV cross-linking step. Therefore, we explored the use of a xenon flash lamp as a source of UV irradiation for cross-linking protein to DNA in very short periods of time (34). The flash lamp produces pulses of UV-rich light in tens to hundreds of microseconds. Figure 3 shows that four consecutive 40-μs flashes provide sufficient cross-linking to detect RNA polymerase II on the *hsp70* gene even in noninduced cells. The 2.2- and 2.4-kb fragments detected in the immunoprecipitates are products of partial digestion and correspond, respectively, to 87A and 87C copies of *hsp70*. Approximately 20-fold more RNA polymerase II is cross-linked to the gene in heat shock-induced cells than in noninduced cells (note that five times as many cells were used to generate the immunoprecipitate from noninduced cells than from heat shock-induced cells).

Figure 4 shows that in noninduced cells a single 40-μs UV flash cross-links RNA polymerase II to an *Ava*I fragment containing the 5′ third but not to one containing the 3′ two-thirds of the *hsp70* gene. The 0.8-, 1.5-, and 3.3-kb fragments contain the 5′ region of the *hsp70* genes (these fragments hybridize to the nick-translated *Sal*I-*Ava*I fragment from the 5′ third of *hsp70*, and the two smallest fragments do not hybridize to nick-translated *Bam*HI-*Sal*I fragment from the 3′ half of *hsp70* [data not shown]). Moreover, the 0.8-kb fragment is the product of complete digestion as predicted on the basis of sequence data and restriction mapping (Fig. 4B) (17, 18). The 1.8-kb fragment is from the 3′ two-thirds of the *hsp70* genes. The total lanes of Fig. 4A provide a measure of the relative amounts of each fragment in the digest to which the immunoprecipitate lanes can be compared. In the total lanes, the 1.8-kb fragment displays the strongest hybridization of these fragments. In contrast, the 0.8-, 1.5-, and 3.3-kb fragments, which contain the promoter region, are detectable in the RNA polymerase II immunoprecipitates, while the 1.8-kb fragment, which contains 1.7 kb of the remaining 3′ region, is not (Fig. 4A, 1 Flash, pol II ppt.). No fragments are detected in the immunoprecipitate when the cells are not irradiated (Fig. 4A 0 Flash, pol II ppt.), nor are the fragments detected when antiserum to *E. coli* RNA polymerase is used for the immunoprecipitation (Fig. 4A, 1 Flash, E. pol ppt.).

DISCUSSION

The in vivo cross-linking studies presented here show that RNA polymerase II is associated with the *hsp70* promoter region in noninduced cells and that most of this polymerase is cross-linked by UV irradiation to an interval from nucleotides −12 to +65. Lower levels of RNA polymerase are detected immediately downstream of +65, but no RNA polymerase is detected on a DNA fragment containing 0.91 kb of the 3′ region of the gene. The association of RNA polymerase II with the 5′ end of *hsp70* is apparent whether the cross-linking is performed by a 10-min UV irradiation of chilled cells with mercury vapor lamps or by a 40-μs irradiation of cells with a xenon flash lamp. Moreover, the amount of RNA polymerase II detected on the promoter of the noninduced gene relative to the amount on the induced gene is the same with either long or short irradiations. Since the reactive species generated by the flash lamp has a half-life of only 40 ms (34), RNA polymerase II has probably not accumulated at the promoter as a result of gene activation by the irradiation. The half-life of the reactive species is short relative to the time expected for gene activation and recruitment of RNA polymerase to a specific promoter (as estimated from procaryotic model systems [6]). We interpret our observations as showing that RNA polymerase II interacts with the *hsp70* promoter even when the gene is not induced.

The amount of RNA polymerase II cross-linked to the noninduced gene is approximately 1/20 the amount cross-linked to the induced gene. In the induced state, approximately 20 molecules are associated with the gene (19), and if we assume that the RNA polymerase II molecules on the noninduced gene cross-link to DNA with an efficiency equal to the RNA polymerase II molecules on the heat shock-induced gene, then we calculate that approximately one molecule is at the 5′ end of the noninduced gene.

Our data do not address the nature of the RNA polymerase II interaction with *hsp70*. It is conceivable that polymerase initiates transcription in the absence of heat shock and that transcription is terminated on average within the first hundred bases of the gene. Premature termination is a commonly found means of transcriptional regulation in procaryotic systems (35). In vitro studies of the *lacUV5* promoter reveal another type of incomplete transcription in which *E. coli* RNA polymerase can cycle producing short (10 base) RNA chains reiteratively without leaving the promoter (5). Several in vitro and in vivo studies have indicated that premature termination may limit the number of full-length transcripts produced from eucaryotic genes as well (1, 21, 31).

Alternatively, RNA polymerase II may simply have a high affinity for the *hsp70* promoter; its release from this high-affinity site could be a critical step in gene activation. In *E.*

coli, the affinity of RNA polymerase for a promoter is an important factor of promoter strength (24), and in vitro, *E. coli* RNA polymerase can bind to the promoter without initiating transcription (6). RNA polymerase II bound to the *hsp70* promoter may be the counterpart of such a complex; however, unlike *E. coli* RNA polymerase, RNA polymerase II does not exhibit specific binding for sequences in naked DNA. Presumably, it requires additional components, perhaps a TATA box-binding protein or a special DNA structure, to direct it to the promoter (4, 28).

The organization of the essential components of the *hsp70* promoter is similar to many eucaryotic promoters. In general, eucaryotic promoters consist of at least two distinct DNA components: the transcription initiation element, which includes the TATA box, and specific regulatory elements (7, 9, 22). The functional and structural similarities between different transcription initiation elements raises the intriguing possibility that the RNA polymerase II interaction with the *hsp70* promoter may be somewhat general. By using a nuclear transcription run-on assay, Gariglio et al. (12) detected RNA polymerase II clustered at the 5′ end of the nontranscribed β-globin gene in nuclei from mature erythrocytes. With our protein-DNA cross-linking method, we have detected a higher level of RNA polymerase II on the copia gene promoter region than on the remainder of the gene in *D. melanogaster* cells (14). Since this gene is transcribed in the cells, it is possible that RNA polymerase rapidly associates with the promoter of this gene but must await a subsequent, rate-limiting event before transcribing the entire gene. In addition, nuclear run-on transcription studies of both the dihydrofolate reductase (2) and c-*myc* genes indicate a higher content of RNA polymerase on the 5′ regions than on the remainder of the genes (2a). These observations indicate that RNA polymerase may be able to interact with the promoters of many genes and that regulation is mediated at steps subsequent to this interaction.

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